Volume 16 Number 6 1988 Nucleic Acids Research

The preparation and application of functionalised synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or -hexanol

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Received December 4, 1987; Revised and Accepted February 25, 1988

ABSTRACT

Syntheses of H-phosphonate salts (4a-e) of N / S-protected alcohols such as 6-aminohexan-1-ol, 3-mercaptopropan-1-ol and 6-mercaptohexan-1-ol are described using 2-chloro-5,6-benzo-1,3,2-phosphorin-4-one (2) as the phosphonylating agent. The H-phosphonate salts (4a-e), in the presence of pivaloyl chloride or adamantoyl chloride as an activator, were coupled to the 5'-end of synthetic oligonucleotides on solid supports to produce amino or thio-linked oligonucleotides. Following deprotection and purification, fluorescent dyes, biotin derivatives and poly-L-lysine-maleimide were separately attached to the functionalised oligonucleotides. Identical derivatized oligomers were obtained with cyanoethyl-N,N-diisopropylamidite chemistry and amidites (5a-e) of the respective alcohols.

INTRODUCTION

Non-radioactive labelling of oligonucleotides and DNA offers an important potential advances to molecular biology methods, medical diagnostics and DNA sequencing[1,11]. In recent years, several methods have emerged for attaching non-radioactive markers. Biotin, one of the most prominent non-radioactive reporter groups, has been incorporated by enzymatic[2-5] and/or chemical[6-9] methods through modified deoxyuridine derivatives. Other synthetic chemical approaches involve the introduction of primary amino[6-11,13-15] or sufhydryl functions[12] to oligonucleotides in the penultimate step of the synthesis followed by addition of fluorophore, chromophore or biotin. To date, reagents for chemically attaching amino or thio functionalised linkers have been developed for use with phosphoramidite and phosphate triester[5,8,20] chemistries. These include N,N-diisopropylamino-phosphoramidites of C-5 modified deoxyuridine[6,16,17], 5'-amino-2',5'-dideoxy uridine[11,19], S-tritylated mercapto alcohols[12], N-protected amino-alcohols[13-15], and 5'-mercapto-2',5'-dideoxy ribonucleotides[18]. Additionally, phosphoramidite derivatives of modified deoxyuridine, 5'-amino-5'-deoxythymidine have been used in lieu of thymidine to result 5'-amino functionalize oligonucleotide. The phosphoramidite derivative of trifluoroacetyl aminoethanol, gives rise to

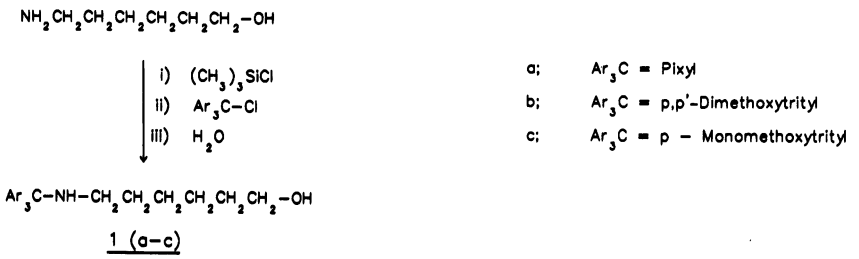
side products and requires additional deprotection steps. The alternative cyclic phosphoramidite[10] derived from trifluoroacetyl aminoethanol requires activation with N,N-dimethylaminopyridine, instead of the commonly used H-tetrazole. Recently phosphoramidites of S - trityl -mercapto -alcohols, S - trityl -5' -mercapto -2',5'dideoxy-ribonucleotides, N-trityl derivatives of aminoalcohols and 5'-amino-2',5'-dideoxyribonucleotides introduced by Connolly and Sproat etal. provide monitoring and purification handle. But no method or reagent is available to functionalise oligonucleotides assembled by the alternative H-phosphonate chemistry[21,22,23], which is also being used for oligonucleotide synthesis.

In this paper, we report the development of a new class of phosphonate functionalization reagents; tertiary ammonium salts of H-phosphonates derived from protected mercapto and amino alcohols. These reagents, in the presence of either pivaloyl or adamantoyl chloride, can be linked to oligonucleotides assembled using either H-phosphonates or phosphoramidite chemistry. The resulting compounds are suitable for conjugation to various reporter groups or to solid supports.

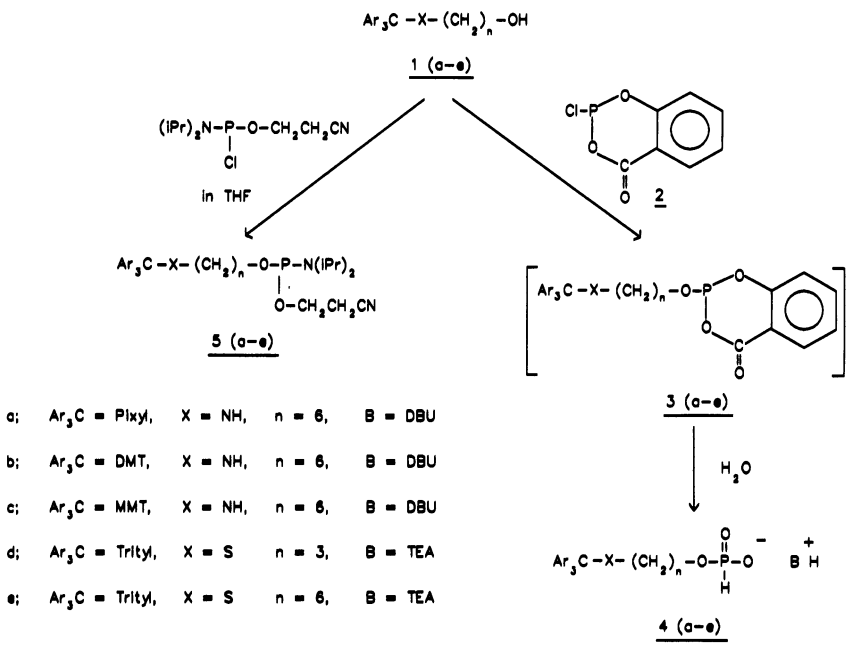
RESULTS AND DISCUSSION

Fluorescent dyes (in the form of isothiocyanate, maleimide, N-hydroxysuccinicimide derivatives) or biotin derivatives (as maleimide or hydroxysuccinicimide) attach efficiently to amino or thiol groups. To introduce these functions to oligonucleotides, we have developed H-phosphonate derivatives (4 a-e) of protected mercapto or amino alcohols (1 a-e). The synthesis involved derivatisation of aminohexanol through transient O-silyl protection as represented in the Scheme. The N-protecting groups used were pixyl, dimethoxytrityl and monomethoxytrityl. Though the synthesis of N-DMT or N-MMT aminohexanol has been reported in the literature[15], the yields based on aminohexanol were low. Our strategy allowed the use of stoichiometric amounts of the trityl chlorides and amino alcohols while minimizing the formation of N,O-ditritylated products. The N-protected alcohols with pixyl (1a) and dimethoxytrityl (1b) were obtained as pale yellow viscous oils but N-monomethoxytrityl aminohexanol (1c) was obtained as a crystalline solid (mp.76-77 ^{o}C). S-Trityl-3-mercapto-propanol (1d) and S-trityl-6-mercapto-hexanol (1e) were synthesised according to the literature[12]. The protected amino and thio-alcohols were converted to their H-phosphonate derivatives using 2-chloro-5,6-benzo-1,3,2-phosphorin-4-one[21] in the presence of diisopropylethylamine in dry THF. The intermediate phosphite triesters (3a-e),

Preparation of N-protected amino alcohols



Preparation of N- and S-protected alkyl H-phosphonate salts



after removal of amine hydrochloride, were treated with water to result in H-phosphonates (4a-e). The crude materials, after purification by silica gel chromatography, were converted into either triethylammonium ($TEAH^+$) or diazabicyclo-undecene ($DBUH^+$) salts using appropriate 0.1 molar bicarbonate solution (pH=7 to 7.5). Usually the triethylammonium salts (4d-e) were obtained as viscous oils whereas the $DBUH^+$ salts (4a-c) were colourless foams. These products were characterised by proton and phosphorus NMR. It is

interesting to note that the $DBUH^+$-phosphonate salts (4a-c) derived from protected alcohols are more stable in solution than their $TEAH^+$ counter parts. The latter compounds tend to decompose by removal of the N-protecting groups on storage in solution. As expected the $TEAH^+$-phosphonates (4d-e) of the S-tritylated mercapto-alcohols were stable for longer periods in solution, because of the greater stability of the S-trityl bond compared to N-trityl bond. N-MMT protection for aminohexanol provided the greatest stability.

These H-phosphonate reagents were incorporated onto oligonucleotides assembled by an automated DNA-synthesiser using H-phosphonate chemistry[21,22,23]. Coupling was performed using pivaloyl chloride in pyridine-acetonitrile (1:1). Adamantoyl chloride was also successfully used as a coupling agent. In the case of thio link introduction, one step oxidation with iodine to convert the synthesised H-phosphonate backbone to phosphate diesters caused removal of the S-trityl group and dimerisation of free thiol-oligonucleotides. Reduction with dithiothrietol was ineffective[12]. The problem of deprotection and dimerisation was circumvented by the use of a two step oxidation procedure. Oligonucleotides synthesised from H-phosphonate precursors were oxidised to phosphate diesters prior to introduction of the thio-linker. After the addition of the thio linker phosphonate, the last phosphonate was oxidized with carbon tetrachloride, triethylamine, N-methylimidazole and water mixture[24]. This step was performed manually after completion of the automated synthesis. Sequences ranging from 11 to 29 bases long were tagged with the linkers. The efficiencies of coupling of the H-phosphonate linkers ranged from 95 to 97%. Cleavage of the N-protected amino-linker tagged oligonucleotides from the solid supports and N-deacylation was carried out at room temperature with aqueous ammonia for 24-36 hr. Higher temperature deprotection caused considerable removal of the N-protecting groups, pixyl being the most and monomethoxytrityl being the least labile. For S-trityl labelled oligonucleotides deprotection cleavage was achieved with aq. ammonia at 55°C for 5-6 hr. On reverse phase HPLC (C-18 column), the desired N- and S-tritylated linker linked oligonucleotides were well separated from the failure and non-nucleotidic organic materials.

The total synthetic material obtained from one micro mole scale syntheses (based on nucleoside originally bound to the CPG-support) were purified by preparative RP-HPLC. The desired pooled fractions were concentrated under reduced pressure. The acid labile protecting groups were removed with 80% acetic acid at room temperature. The S-trityl was cleaved with silver nitrate solution (1.0 M, 5.0 eq.) and silver ion was removed from the mixture with

dithiothreitol[12] (DTT) to give free sulfhydryl linked oligonucleotides. Incorporation of the linkers were confirmed independently by synthesising these oligonucleotides from phosphoramidites of deoxynucleosides[25] and N- or S- protected amino or thio alcohols (1a-c, 1d-e). Some of these phosphoramidites (5c-e) of N- or S-protected alcohols are new compounds (see methods and materials) and have been characterised by ^{31}P NMR. RP-HPLC analysis and electrophoretic mobilities of these and those obtained from phosphonate chemistry were identical.

The purified free amino or thio-linked oligonucleotides were suitable for subsequent dye or non-radioactive marker incorporation following the published procedures[12,14,15]. The thio-link oligonucleotides were coupled to monobromo bimane, eosin-maleimide, 1,5-I-AEDANS, 7-N,N-diethylamino-4-methyl-3-(4'-maleimidylphenyl)-coumarin and biotin-maleimide. The amino-linked derivatives were attached to fluorescein isothiocyanate, nitro-benzo-diazole fluoride and biotin-N-hydoxysuccinicimide. Also a thio-linked oligonucleotide was conjugated to poly-L-lysine-maleimide derivative (see methods and materials section). This method is much simpler than the published procedure[26]. We are also interested in coupling thio-linked oligonucleotides onto solid supports for affinity chromatographic application and this work is currently under investigation.

METHODS AND MATERIALS

Aminohexanol, triphenylmethyl mercaptan, trimethylsilyl chloride, dimethoxytrityl, monomethoxytrityl and pixyl chlorides were purchased from Aldrich. Fluorescent dyes and poly-L-lysine, biotin-maleimide, N-hydroxysuccinicimide of caproyl-maleimide were procured from Molecular Probes and Sigma chemical companies respectively. The H-phosphonate nucleoside precursors[21,23] and beta-cyanoethoxy monochloro N,N-diisopropylamino phosphine[25] were synthesised according to literature methods. Thin layer chromatography (TLC) was performed with precoated silica gel on aluminium plate (E. MERCK) using a) ether-hexane or b) ethyl acetate or c) methylene chloride-methanol mixture (8.5:1.5 v/v).

HPLC analysis or purification was carried out on Varian 5000 instrument and RPC-18 column. A gradient of acetonitrile (0 to 35% or 0 / 5 to 40%) in either triethylammonium acetate or bicarbonate buffer (0.1 M, pH=7 to 7.5) was used. Gel filtration chromatography was performed over Sephadex G 25-50 using HPLC grade water as a eluent. Gel-electrophoresis were usually run on 20% polyacrylamide gel containing 7.0 M urea.

Synthesis of N-protected aminohexanols (1a-c):
Dry aminohexanol (50.0 mmol) was dissolved in purified and dried pyridine (100 ml) and treated with freshly distilled chloro trimethylsilane (150 mmol) at 0°C. Pixyl/dimethoxytrityl/ monomethoxytrityl chloride dissolved in dry pyridine (50 ml) was added to this mixture after 30 min. and stirred at room temperature. The reactions were monitored by TLC. After 2 to 3 hr.of stirring, the excess chloro trimethylsilane and pyridine were removed on rotary evaporator to result in a viscous oil. This viscous oil was taken up in ether (500 ml) and washed with ice cold $NaHCO_3$ solution. Ether extract dried over anhydrous Na_2SO_4 was concentrated down to yellow oils. The crude materials were purified by silica gel chromatography using hexane-ether mixture containing small amount of pyridine (0.5%). The O,N-di pixyl/DMT/MMT-aminohexanol (3 to 7%), in each case eluted in the first few fractions (15 to 20% ether). N-Protected (trityl or pixyl)-aminohexanol eluted at greater percentage of ether and was the major product (75 to 85%). N-Pixyl and N-dimethoxytrityl-aminohexanols were obtained as pale yellow oils, attempted crystallisation was unsuccessful. N-monomethoxytrityl-aminohexanol, after chromatographic purification, crystallised from hexane-ether (1:1) mixture as a white crystal (mp.76-77°C).

General method for preparation of tertiary ammonium salt of H-phosphonate (4a-e):
The dried N/S-protected alcohol (20 mmol) was dissolved in a mixture of dry THF (50 ml) and diisopropylethyl amine (55 mmol). To this,a solution of 2-chloro-5,6-benzo-1,3,2-phosphorin-4-one (25 mmol) in THF (25 ml) was added dropwise over 15 min under argon atmosphere. The insoluble amine-hydrochloride formed was filtered after 45 min of stirring. The clear solution so obtained was treated with cold water (5 ml) to result H-phosphonate monoester. The solvent THF was removed under reduced pressure.The concentrated yellow mass was dissolved in ethyl acetate (250 ml) and extracted with triethylammonium bicarbonate buffer (0.1 M, pH=7.5). The crude material was purified by silica gel chromatography using methanol (5 to 20%) in methylene chloride containing 0.1% of triethylamine. The desired materials, being polar, were easily isolated from starting materials and byproducts. The isolated yields of S-trityl-mercapto-alkyl-H-phosphonate triethylammonium salts (as oils) ranged 75 to 85%, and those of N-protected aminohexyl-H-phosphonate triethylammonium salts (also as oils) were 65 to 75%. The latter TEA-salts were changed into more stable diazabicyclo-undecene salts by treating the methylene chloride solutions with DBU-bicarbonate (0.1 M,pH=7.5) and were obtained as solid foams.

General method for Synthesis of the beta-Cyanoethyl-N,N-Diisopropylamino Phosphoramidites from N/S-protected Alcohols (5a-e):

The beta-cyanoethyl-N,N-diisopropylamino phosphoramidites of N- / S-protected alcohols were prepared adapting the method used for synthesis of protected deoxynucleosides-phosphoramidites[25]. The protected alcohols (10 mmol) in dry THF (50 ml) was generally allowed to react with monochloro-beta-cyanoethoxy-N,N- diisopropylamino phosphine (12.5 mmol) in the presence of diisopropylethylamine (30 mmol). After work up under argon atmosphere, the crude materials were purified over silica gel chromatography using ether-hexane mixture to result the amidites as clear oils (yields 75 to 90%). The purity of these compounds was checked by ^{31}P NMR.

Syntheses of Amino-Linked Oligodeoxynucleotides using N-tritylated aminohexyl H-phosphonate salts (4a-c):

Several oligodeoxynucleotides (11 to 29 bases long) were assembled on a DNA-synthesiser using 1 umole nucleoside bound supports and H-phosphonates of deoxynucleosides. At the end of desired sequence assembly, a solution of DBU-salt of tritylated aminohexyl-H-phosphonate [25 mg/ml of a mixture of pyridine-acetonitrile (1:1)] was passed through the support followed by alternatively passing a pivaloyl chloride (0.2 M) soln.in acetonitrile-pyridine (1:1). The coupling efficiency of amino-linker was estimated colorimetrically by the release of tertiary carbonium ions with 10% TCA in CH_2Cl_2. The H-phosphonate esters present were oxidised to the phosphate diesters with a cock-tail of iodine: THF: water: N-methylimidazole: (1g:18ml:7ml:1ml) for 40 minutes. These steps were controlled by DNA-synthesiser. The cleavage of the synthetic oligonucleotide from the support and concomitant N-deacylation were carried out by incubating the support in aqueous ammonia (29%) for 24 to 36 hr. at room temperature. After usual work up[25], the crude synthetic material was taken up in a triethyl ammonium bicarbonate solution (0.1M,pH=7.5;1.0 ml). and analysed by RP-C 18 HPLC using acetonitrile in TEAB soln. The total synthetic material was purified by preparative C-18 column using step linear gradient of acetonitrile in TEAB buffer (8ml/min.);[i. 0% CH_3CN for 10 min,ii. 0 to 25% CH_3CN in 10 min,iii. 25% CH_3CN for 10 min,iv. 25 to 40% in 10 min, and v. 40% for 5 to 10 min.). The purity of free amino-linked oligonucleotide obtained after detritylation with 80% acetic acid was checked by analytical anion exchange or reverse phase HPLC.

Incorporation of aminohexyl-O-phosphate linker at the 5'-end was confirmed by

synthesising two oligonucleotides (aminohexyl- linked oligonucleotide 11-mer and aminohexyl-linked oligonucleotide 15-mer) using beta-cyanoethyl-N,N-diisopropyl- amino phosphoramidite of the deoxynucleosides and N-MMT aminohexanol (1c).

Synthesis of S-tritylated Thio-Linked Oligonucleotides using Triethylammonium salts of H-phosphonates (4d-e):

The desired oligonucleotides were synthesised on solid support (0.2 to 1.0 u mol scales) following phosphonate chemistry. The resulting internucleotidic H-phosphonate linkages were converted into phosphate diesters linkage with iodine mixture. The desired thio-linker was introduced to the oligonucleotide via H-phosphonate chemistry, by pulsing alternatively, a solution of S-trityl-mercaptopropanol-/-hexanol-H-phosphonate in pyridine-acetonitrile and an activator solution through the support. After synthesis, the column containing the support was treated with a mixture of carbon tetrachloride: triethylamine: N-methylimidazole (9:0.5:0.5 v/v) containing 10% water for 10 min to convert the last introduced H-phosphonate moiety into a stable phosphate diester. The S-tritylated-thio-linked oligonucleotide was separated from the support by treating with aqueous ammonia at 55°C for 5-6 hr. The synthesised material was analysed and purified by RP-HPLC. Trityl was removed with silver nitrate solution (1.0 M,5.0 eq.) for 20 min and free sulfhydryl-linked oligonucleotide was obtained by centrifugation with dithiothreitol (1.0 M,7.0 eq.). The excess of DTT was removed with ethyl acetate under argon. Formation of the desired thio-linked oligodeoxynucleotide was confirmed by synthesising these oligonucleotides from beta- cyanoethyl- N,N- diisopropylamino phosphoramidites (5d,e) of S-tritylated-mercapto-propanol or corresponding hexanol and deoxynucleoside amidites. The S-tritylated-thio-linked oligonucleotides obtained from the different routes had the same retention time and similar chromatogram profiles.

Attachment of S-Trityl-6-mercapto-hexyl-H-phosphonate (4d) or N-Monomethoxytrityl-aminohexyl-H-phosphonate (4c) onto oligonucleotides assembled by the phosphoramidites chemistry:

An oligodeoxynucleotide of 21 bases long was synthesised (1 umol CPG) using phosphoramidite methodology. The 5'-dimethoxytrityl protecting group was removed and the support was dried with argon. A mixture of H-phosphonate salt ($TEAH^+$ or $DBUH^+$; 25 mg) of S-trityl-6-mercaptohexanol or N-MMT-6-aminohexanol and pivaloyl chloride (0.2 M) in pyridine-acetonitrile (1:1;2 ml) was passed through the column with a syringe over 3 to 5 minutes. After oxidation with

either iodine solution or aqueous carbon tetrachloride mixture, the oligomers were cleaved from the support and concomitantly N-deacylated with ammonia at room temperature for 24 hr. The desired N-MMT-aminohexyl or S-tritylmercaptohexyl-5'-phosphate-oligodeoxynucleotide (21-mer) were present in good yield (60% of the crude sample by RP-HPLC).

Attachment of Fluorescent dyes onto the 5'-end of derivatised oligonucleotides:

a) To a sulfhydryl-linked oligomer: Incorporation of thiol specific dyes were achieved according to the literatures[12,15]. The products were purified by gel filtration chromatography using Sephadex G 25-50. Fluorophores used for tagging the sulfhydryl moiety are the following:

i) Monobromobimane, ii) Eosin-maleimide, iii) 1,5-I AEDANS, iv) 7-N,N-Diethylamino-4-methyl-3-4'-maleimidophenyl)-coumarin. Eosin and AEDANS tagged materials had to be purified extensively and unlike others, decomposed on storage. A biotin maleimide derivative was also conjugated.

b) To the aminolinked oligonucleotides: FITC, NBD-fluoride or biotin derivatives attached to aminolinked oligonucleotides following the published procedure[14]. The yield of dye incorporated oligonucleotides was found to be more than 70%.

Preparation of Oligodeoxynucleotide-poly-Peptide-Conjugate:

a) Synthesis of poly-L-lysine maleimide derivative: This compound was prepared by adaptation of the literature procedure[27]. To a solution of poly-L-lysine (140 mg,10 umol) in a HPLC grade methanol (5 ml), was added maleimido-caproyl-N-hydroxysuccinic-imide ester (5 mg) and triethylamine (200 ul). After 3 hr of stirring at room temperature, N-hydroxysuccinimide generated was removed by centrifugation. The clear liquid was concentrated to result a white solid. The solid was stored under argon at -22°C.

b) Conjugation of sulfhydryl linked oligonucleotide to poly-l-lysine maleimide derivative: Freshly generated thio-linked 21-base oligomer (10 OD in 250 ul water), was treated under argon atmosphere with poly-L-lysine-maleimide compound (12.0 mg) at room temperature for 20 hr. The reaction mixture was loaded on Sephadex-G25-50 column (2.0 x 25 cm) and eluted with water. Fractions of 300 to 400 ul volume were collected and checked for oligonucleotide and peptide content by measuring absorbances at 254 and 230 nm respectively. Fractions with positive readings at both wave lengths were combined.

CONCLUSION

A class of reagents based on H-phosphonate chemistry, allowing the introduction of amino or thio functionality onto synthetic oligonucleotides, is described.

The reagents are readily prepared,purified, stable and easy to handle. The H-phosphonate reagents have been linked both to oligonucleotides synthesised from H-phosphonate and from phosphoramidite precursors. Monomethoxytrityl as N-protection was preferred since it was unaffected by deprotection and purification conditions. The trityl group used for S-protection was unaffected under deprotection or purification conditions and was selectively removed to generate free thiol linked oligonucleotide.

Although only C_3 and C_6 alcohols have been used the methods are general and suitable for other aliphatic amino or thio-alcohols. The ability to conjugate oligonucleotides to reporter groups should be greatly enhanced utilizing the described reagents.

ACKNOWLEDGEMENT

Authors sincerely thank Dr. M.H. Lyttle, Mr. P. Wright and S. Streipeke for their help in carrying out some oligonucleotide syntheses used in this work and Drs. D. Hudson and J. Giles for their suggestions and help during the preparation of this manuscript.

ABBREVIATIONS

1,5-IAEDANS = 5-[2-{(Iodoacetyl)amino}ethyl]-aminonaphthalene-1-sulphonic acid

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